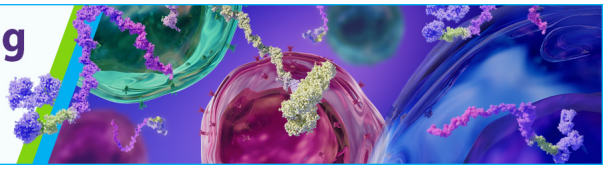

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Secondary Lymphoid Tissue Chemokine (CCL21) Activates CXCR3 to Trigger a Cl^- Current and Chemotaxis in Murine Microglia[1]

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Microglial cells represent the major immunocompetent element of the CNS and are activated by any type of brain injury or disease. A candidate for signaling neuronal injury to microglial cells is the CC chemokine ligand CCL21, given that damaged neurons express CCL21. Investigating microglia in acute slices and in culture, we demonstrate that a local application of CCL21 for 30 s triggered a Cl^- conductance with lasted for tens of minutes. This response was sensitive to the Cl^- channel blockers 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and 4-acetamide-4′-isothiocyanatostilbene, 2,2′-disulfonic acid. Moreover, CCL21 triggered a chemotaxis response, which was sensitive to Cl^- channel blockers. In microglial cells cultured from CCR7 knockout mice, CCL21 produced the same type of Cl^- current as well as a chemotaxis response. In contrast, in microglial cells from CXCR3 knockout mice, CCL21 triggered neither a Cl^- conductance nor a chemotaxis response after CCL21 application. We conclude that the CCL21-induced Cl^- current is a prerequisite for the chemotaxis response mediated by the activation of CXCR3 but not CCR7 receptors, indicating that in brain CCL21 acts via a different receptor system than in lymphoid organs. ***The Journal of Immunology,*** **2002, 169: 3221–3226.**

Microglial cells, brain-specific macrophages, are viewed as the major immunocompetent elements of the CNS (1, 2). Although their immune functions are downregulated in the healthy brain, microglia rapidly activate upon inflammatory processes, chronic brain disease, or brain injury. Microglial activation is thus a general response to pathological processes in the CNS in which the ramified microglia transform into an ameboid macrophage like phenotype (3, 4). Because microglia are the first cells in the CNS to respond to neuronal damage, a neuron-microglia communication system has been proposed (4, 5). One candidate for such a signaling molecule is an α chemokine ligand (CCL21)[3]; damaged neurons in vitro and in vivo rapidly induce CCL21 expression (6). Because CCL21 induces intracellular calcium signals and chemotaxis of cultured microglia, a potential role of CCL21 in neuron-microglia communication has been proposed (6). Electrophysiological responses are the most rapid form of cellular communication, and we have therefore used the patch-clamp technique to record changes in membrane currents of microglia in response to CCL21. The membrane channel pattern of microglial cells is unique for CNS cells and furthermore different with respect to non-CNS macrophages. Therefore, this physiological fingerprint is used to identify microglial cells in culture and brain slices (7).

CCL21 (formerly known as secondary lymphoid organ chemokine, TCA4, 6Ckine or exodus-2) is a lymphoid chemokine and is constitutively expressed in secondary lymphoid organs. It controls the homing of naive T cells and mature dendritic cells, all of which express the corresponding receptor CCR7 (8, 9). Other chemokines are inflammatory like CXCL10 (formerly known as IFN-γ-inducible protein 10), which is induced under inflammatory conditions and controls the infiltration of the inflamed tissue by CXCR3-expressing lymphocytes (10). Chemokines mediate their biological activities through G-protein-coupled cell surface receptors of the seven-transmembrane domain, rhodopsin-type superfamily (11, 12). As in the periphery, chemokines are also highly expressed in CNS inflammation (13). According to their function in the peripheral immune system, CNS chemokines have been suggest to orchestrate the infiltration of the CNS by blood leukocytes (14, 15), given that it is well known that all intrinsic brain cells (neurons and glial cells) express chemokine receptors (14). Therefore, several authors (13, 16, 17) have suggested a possible role for chemokines in intercellular signalling in CNS.

Materials and Methods

Cell culture preparation

Microglial cells were prepared and purified from primary cultures of newborn NMRI mouse brains essentially as described previously (18, 19). In brief, brains were removed under sterile conditions from the skull and meninges, and blood vessels were carefully removed. The total brain was trypsinized for 5 min. After centrifugation, the pellet was resuspended in FCS-containing DMEM and washed twice. Finally, the cell suspension was plated in poly-L-lysine-coated tissue culture flasks (cells from two brains per flask). Thereafter, the medium was changed after 24 h and then every third day.

After 7–10 days in culture, microglia were detached from the astrocytic monolayer by manually shaking the cultures for 2–3 min. For patch-clamp

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[3] Abbreviations used in this paper: CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; SITS, 4-acetamido-4′-isothiocynatostilbene-2,2′-disulfonic acid; [Cl^-], chloride concentration.

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experiments, isolated microglia were seeded on glass coverslips at a non-confluent density of $3 \times 10^4/cm^2$. Electrophysiological recordings were performed 1–5 days after the isolation procedure. For chemotaxis assays, detached microglial cells were washed once and directly used. Cell media and supplements were purchased from Seromed/Biochrom (Berlin, Germany).

CCR7 and CXCR3 knockout (−/−) mice

Homozygotes of the CCR7 and CXCR3 knockout line were used. Microglial cultures were prepared from CCR7 and CXCR3 knockout mice as described above. Generation of the knockout lines have been described previously (20, 21).

Drugs and solutions

HEPES-buffered salt solution contained 150 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 10 mM glucose, adjusted to pH 7.4 with NaOH. The bicarbonate-buffered salt solution contained 134 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.25 mM K_2HPO_4, 26 mM $NaHCO_3$, and 10 mM glucose. The recombinant CCL21 and CXCL10 from PeproTech EC (London, U.K.) and CCL19 from R&D (London, U.K.) were prepared as 10 μM stock solutions in PBS. The human recombinant C5a was purchased from Sigma (Deisenhofen, Germany) and prepared as a 2×10^{-4} M stock solution in PBS and 0.1% BSA. For the chloride substitution experiments, NaCl was replaced by equimolar amounts of 500 μM sodium gluconate and 1 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocynatostilbene-2,2′-disulfonic acid (SITS) were dissolved in the HEPES bath solution. The pipette solution contained 130 mM KCl, 0.5 mM $CaCl_2$, 3 mM $MgCl_2$, 10 mM HEPES, and 5 mM EGTA, adjusted to pH 7.3 with KOH.

Microchemotaxis assay

Cell migration assays in response to CCL21 were performed in a 48-well microchemotaxis chamber (Neuroprobe, Bethesda, MD) as previously described (22). Briefly, lower wells were loaded with chemokines diluted in serum-free DMEM. DMEM was used as a negative control, and 100 nM C5a was used as a positive control. Upper and lower wells were separated by a polycarbonate filter (8-μm pore size; Poretics, Livermore, CA). Microglial cells ($2–3 \times 10^4$) in 50 μl serum-free DMEM medium were added to the upper wells, and the chamber was incubated at 37°C and 5% CO_2 for 120 min. Experiments were performed in triplicates. Rate of microglial migration was calculated by counting cells in four random fields of each well using a ×40 bright field objective. The chemokine-induced migration was normalized for migration of the unstimulated control groups and presented as percent of controls ± SEM. Significance levels were calculated using a one-way analysis of variance (*t* test). A value of $p < 0.01$ was considered significant.

Electrophysiology

Glass coverslips with adherent microglial cells were placed in a recording chamber on a microscope equipped with phase-contrast optics (Axiovert FS; Zeiss, Oberkochen, Germany). The chamber was permanently perfused with bath solution, and all patch-clamp recordings were made at room temperature. Voltage-clamp experiments were conducted using the whole-cell variant of the patch-clamp technique (23). Microelectrodes with 5–10 MΩ resistance were fabricated from thin-walled borosilicate capillaries with filament. Uncompensated whole-cell currents were recorded with an EPC-9 patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany) at a sampling rate of 3 kHz. Signals were filtered at 2.9 kHz. Voltage-clamp protocol, data acquisition, storage, and analysis were controlled by software (Wintida 4.02; HEKA). Low Cl^-, SITS, and DIDS were applied by changing the bath solution. For Cl^- current recordings, the bath electrode was separated with an agar bridge of 1% agar from the bath solution. To stimulate microglial cells with CCL21 during patch-clamp recordings, we used a second application pipette, which was filled with 10 μM CCL21 and placed close to the cell. CCL21-containing solution was ejected by applying pressure to the rear of the pipette. As a control for the application system, PBS was ejected from a similar type of micropipette; only in 1 of 18 cells was a change in membrane conductance observed.

The preparation of acute brain slices of 6-wk-old mice was as described previously (24). Briefly, 130-μm-thick slices were cut with a vibratome (Vibracut; FTB Feinwerktechnik, Bensheim, Germany) in ice-cold bicarbonate-buffered salt solution gassed with carbogen (5% CO_2 and 95% O_2). For identification of microglial cells, slices were stained for 45 min at 37°C with 100 μg/ml Texas Red-coupled tomato lectin (Sigma).

RT-PCR

Cells and brain material were lysed in guanidinium isothiocyanate-mercaptoethanol buffer, and total RNA was extracted with slight modifications according to the method of Chomczynski and Sacchi (25).

Reverse transcription. One microgram of total RNA was transcribed into cDNA as described (26). Potential contaminations by genomic DNA were checked by running the reactions without reverse transcriptase and using GAPDH primers (housekeeping gene) in subsequent PCR amplifications. Only RNA samples that showed no bands (no contamination) were used for further investigation.

PCR. Two microliters of the RT reaction were used in subsequent PCR amplification as described (26). Cloning into pCRII (Invitrogen, San Diego, CA), and subsequent sequencing verified the identity of the PCR products.

Results

CCL21 triggers a membrane conductance in microglial cells in culture and acute brain slices

To test for the presence of functional CCL21 receptors on cultured microglial cells, we used the patch-clamp technique to analyze conductance changes after applying the ligand CCL21. To obtain CCL21-induced current changes, we repetitively clamped the membrane at a holding potential of −20 mV for a series of voltage steps ranging from −120 to +60 mV (100 ms/step, 100-ms interval, 20-mV increment, 150-Hz sampling frequency, every 5 s). CCL21 was applied by pressure ejection from a micropipette, which was placed close to the cell (30-s application, 10 μM). In 12 of 21 cells, CCL21 activated a long lasting membrane conductance (Fig 1A). This current activated slowly and continued to increase after CCL21 had already been washed out. The CCL21-induced membrane conductance (average, 5.2 ns) reached its peak after ~5 min, and returned to its resting level 10 min after the application of CCL21. To isolate the CCL21-induced current at high time resolution (3-kHz sampling frequency), we repetitively clamped the membrane for 50 ms at a holding potential of −20 mV for a series of nine depolarizing and hyperpolarizing voltage steps with an increment of 10 mV. The current-voltage curve of the CCL21-induced current (Fig. 1*A*) showed an outward rectification and a reversal potential close to 0 mV. The current did not inactivate. This suggests the involvement of either a nonselective cation conductance or a chloride conductance. In only 4 of 12 cells, could a second CCL21 response be elicited; furthermore, the conductance increase induced by the second application was smaller than that of the first (data not shown).

To investigate the expression of functional CCL21 receptors in microglia cells in situ, we used acutely prepared coronal slices from 8-wk-old mice. Before recording, microglial cells were identified by staining the slice with tomato lectin, and a similar recording protocol was applied as used for the microglia in culture. CCL21 triggered a similar type of membrane conductance in 6 of 7 cells (Fig. 1*B*). This increase in membrane conductance was, however, not reversible within the recording time of about 1.5 h.

CCL21 triggers a long lasting Cl^- conductance

To test for the ionic specificity of the CCL21-induced current, the Cl^- gradient across the membrane was altered by decreasing extracellular Cl^- concentration ($[Cl^-]$) from 161.4 to 6 mM by equimolar substitution of Cl^- with gluconate. CCL21 was first applied and washed out; subsequently the Cl^- gradient was changed for 2 min while the membrane conductance slowly increased (Fig. 2*A*). In low Cl^-, the membrane conductance decreased, mainly affecting the outward conductance. Concomitantly, the reversal potential shifted to a more positive value, namely to +40 mV on average (Fig. 2*B*). Thus, the reversal shifts toward the new, theoretical Cl^- equilibrium potential (+80 mV),

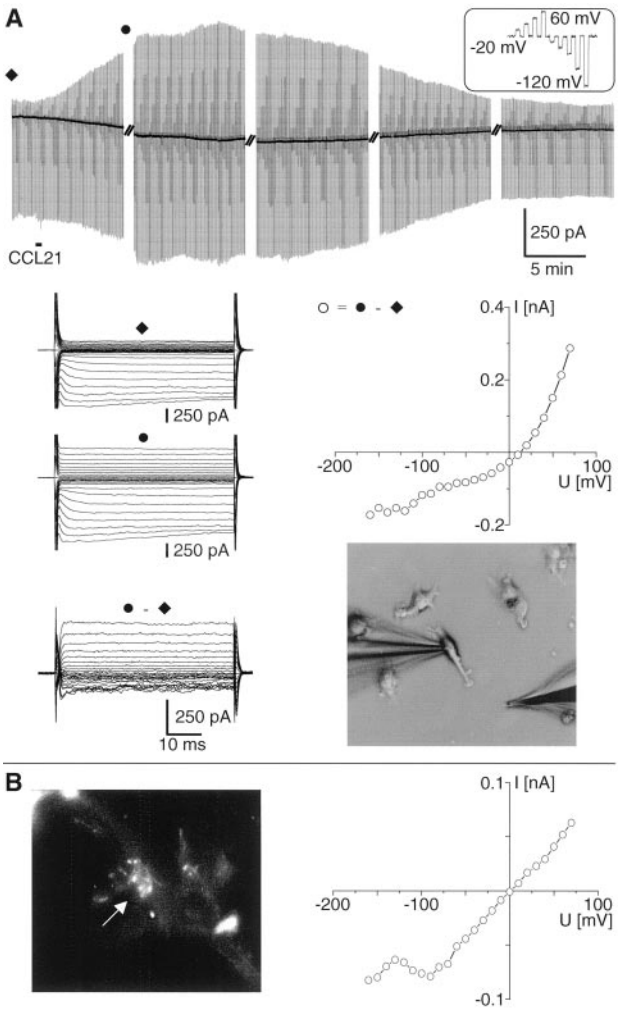

FIGURE 1. Induction of CCL21-activated currents in mouse microglial cells. *A*, From a holding potential of −20 mV, the membrane of cultured microglial cells was clamped with 20-mV increment from −160 mV to +60 mV for 100 ms. This protocol was applied repetitively every 5 s. CCL21 (10 μM) was applied for 30 s as indicated by the bar. The single 7 voltage steps cannot be resolved at this resolution, but the slow increase in membrane conductance is apparent. To record membrane currents with high time resolution, the continuous recording was interrupted for 1 min (‖), and the membrane was clamped from −160 mV to +70 mV with 10-mV increment. The three series of current traces on the left represent currents preceding CCL21 application (*top*, ◆), after the onset of the conductance increase (*middle*, ●), and the CCL21-induced current (*bottom*, ●–◆). The latter was obtained by the subtraction of the currents measured before CCL21 application and after CCL21 application. The corresponding current to voltage curve of the CCL21-induced current is shown on the right. Inset, Experimental arrangement. The recording pipette is attached to a microglial cell (*left*), and CCL21 was ejected from the pipette on the right. *B*, A ramified microglial cell close to a blood vessel was identified by its positive label for tomato lectin (*left*, fluorescence image; arrow, microglial soma). Similarly, as shown for the cultured cell in *A*, the current voltage curve of the CCL21-induced current was recorded and is displayed in the current to voltage curve on the right.

yet it is still more negative. This discrepancy can be explained due to either decreased intracellular Cl^- activity or a significant permeability to gluconate as described by Xu et al. (27).

To further substantiate the Cl^- selectivity of the CCL21 induced current, we tested the effect of the Cl^- channel blockers SITS and DIDS at both 500 μM and 1 mM. After triggering an increase in membrane conductance with a brief CCL21 application, DIDS and SITS were applied via the bath solution. Both chloride channel blockers reversibly reduced the currents by 81 ± 5.78% for 1 mM SITS (Fig. 2, $n = 8$) and by 79 ± 4.67% for 1 mM DIDS (not shown, $n = 5$). The blockade was more pronounced with positive membrane potentials. SITS or DIDS (500 μM) also reduced the CCL21-induced current, but slightly less effectively.

CCL21 and CXCL10 act via CXCR3 receptors

CCL21 commonly acts via CCR7 receptors (28–30). We therefore used another CCR7 ligand and studied the effect of CCL21 on membrane currents in microglial cells derived from mice in which the CCR7 locus has been disrupted by gene targeting. Stimulation of microglia with CCL19 did not induce any change in membrane conductance ($n = 27$, data not shown). Moreover, in microglial cells derived from CCR7 knockout animals, CCL21 triggered an increase in conductance similar to that in control cells. These results indicate that CCR7 is not the receptor for CCL21 in microglia (Fig. 3*A*; $n = 17$).

There is recent evidence that microglia express the chemokine receptor CXCR3 (6). We therefore tested the effect of CCL21 in microglial cells cultured from CXCR3 knockout animals. CCL21 did not trigger any membrane conductance change, indicating that CXCR3 is a microglial receptor for CCL21 (Fig. 3*A*; $n = 21$).

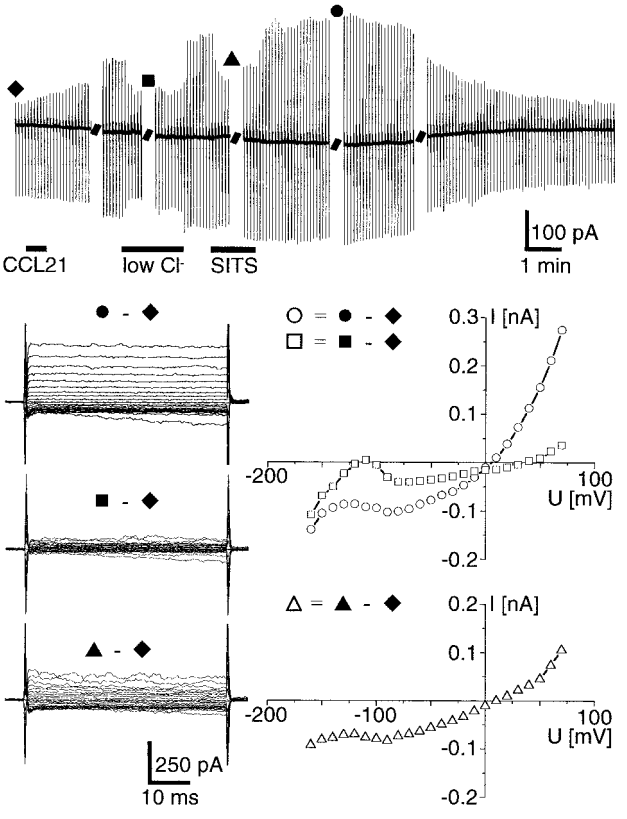

FIGURE 2. Influence of extracellular chloride concentration and the chloride channel blocker SITS on CCL21-activated currents. The experimental procedure is as described in Fig. 1. CCL21 (10 μM, 30 s), low Cl^- (6 mM, 2 min), and SITS (500 μM, 90 s) were applied as indicated by bars (holding potential, −20 mV). The extracellular [Cl^-] was reduced from 161.4 mM to 6 mM by equimolar substitution of gluconate. The series of current traces *(left)* displays the CCL21-induced currents (obtained as described in legend to Fig. 1) at the peak of the response (*top*, ●–◆), in low Cl^- (*middle*, ■–◆), and in the presence of SITS (*below*, ▲–◆). The corresponding current to voltage curve is shown on the *right*.

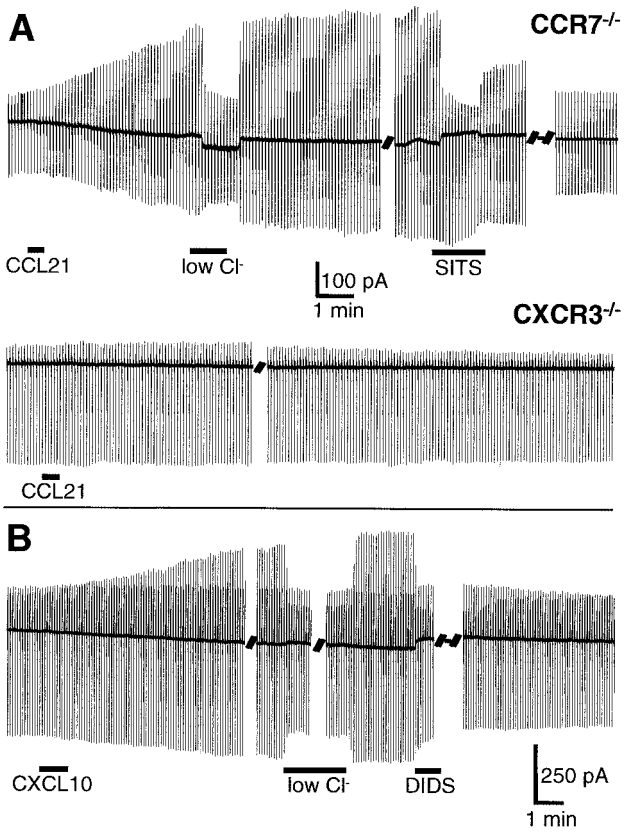

FIGURE 3. Identification of CXCR3 as the receptor for CCL21. *A*, CCL21-induced currents in microglial cells obtained from CCR7 knockout mice *(top)* and CXCR3 knockout mice *bottom*. CCL21 (10 μM) and SITS (1 mM) were applied as indicated. Low Cl^- represents a reduction in [Cl^-] to 6 mM. ||-||, Interruption of 7 min. *B*, Similarly, the effect of the chemokine CXCL10 was tested, with the Cl^- channel blocker DIDS (1 mM). ||-||, Interruption of 8 min.

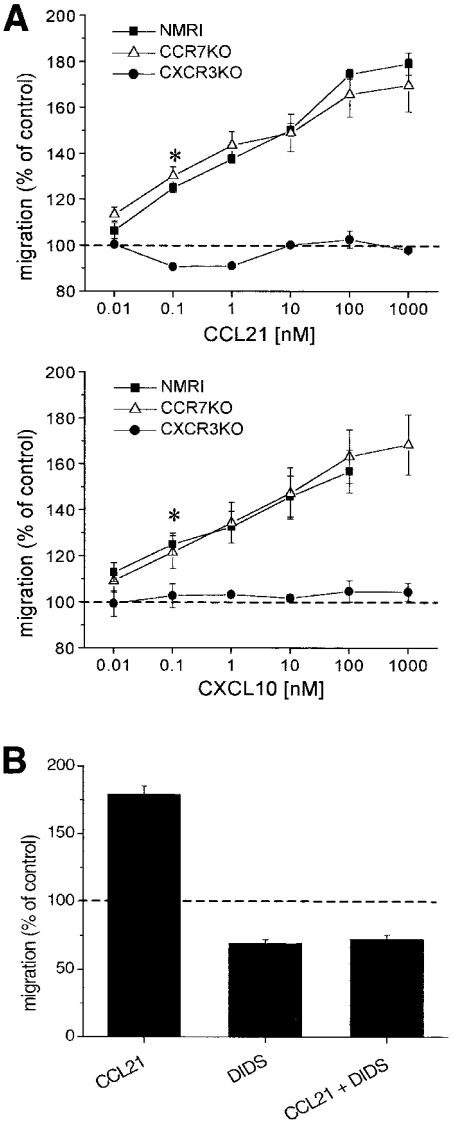

FIGURE 4. CXCR3 activation by CCL21- and CXCL10-triggered microglial migration. *A*, Migration rate of microglial cells cultured from wild-type (NMRI), CCR7 knockout (CCR7KO), and CXCR3 knockout (CXCR3KO) animals in response to increasing concentrations of CCL21 and CXCL10. Chemokine-induced migration was normalized to the unstimulated control (±SEM). The graph demonstrates the mean of $n = 4$–7 independent experiments. Significant migration above control (DMEM) was calculated using a one-way analysis (Student *t* test) of variance. *, Significant migration above control levels ($p < 0.01$). *B*, Migration of microglial cells in the presence of CCL21 (100 nM), CCL21 and DIDS (1 mM), and DIDS alone are shown and compared with the control.

To further confirm the expression of CXCR3 in microglia, we tested microglial responses to CXCL10 (formerly known as IFN-γ-inducible proteins 10), one of the high affinity ligands for the CXCR3. CXCL10 was applied in paradigm comparable with that of CCL21 and triggered a similar long lasting membrane conductance increase similar to CCL21 (Fig. 3*B*; $n = 11$). The CXCL10-induced current shared all the features with the CCL21 induced current: 1) it reversed close to 0 mV in normal bathing solution; 2) lowering of the extracellular Cl^- concentration shifted the reversal potential of this current to more positive potentials, and led to a reduction in the current amplitude; 3) the chloride channel blockers DIDS and SITS reversibly reduced the CXCL10-induced current; 4) a CXCL10-induced current was observed in microglial cells from CCR7 knockout ($n = 9$), but 5) not in those from CXCR3 knockout animals ($n = 17$).

Chemotaxis is controlled by CXCR3 receptors and impaired by Cl^- channel blockers

To test for the involvement of CXCR3 receptors in microglial chemotaxis, both the effects of CCL21 and CXCL10 in an in vitro chemotaxis assay were investigated. Under control conditions microglial cells showed considerable random migration. Both chemokines (Fig. 4) induced a significant increase in chemotaxis of cultured microglia compared with unstimulated control cells at a concentration range of 0.1 nM–1 μM ($p < 0.01$). CCL21 (100 nM) and CXCL10 (100 nM) led to an increase in infiltrating cells by 175 ± 2.10% (four independent experiments) and 157 ± 9.33% ($n = 5$), respectively, as compared with controls. The chemotactic migration of cultured microglial cells from CCR7 knockout animals in response to 100 nM CCL21 and 100 nM CXCL10 was comparable with that of wild-type animals, namely 165 ± 9.96% for CCL21 ($n = 7$) and 163 ± 11.65% for CXCL10 ($n = 6$). In contrast, in microglial cells cultured from CXCR3 knockout animals, both chemokines did not significantly increase the number of cells that migrated into the filter, indicating that these chemokines did not stimulate chemotactic migration (Fig. 4, $n = 5$). However, the chemotactic behavior in general was not impaired in these knockout mice, because microglial migration in response to C5a was found (data not shown). Taken together, the results indicate

that activation of CXCR3 by CCL21 or CXCL10 stimulate the chemotactic behavior of microglial cells.

To test for a possible interaction between Cl^- conductance and chemotaxis, we examined the effect of the Cl^- channel blockers DIDS in the chemotaxis assay at the same concentration in which it effectively blocked the CCL21-induced Cl^- conductance. Stimulation of microglial migratory activity by CCL21 (100 nM) was absent in the presence of 1 mM DIDS (Fig. 4*B*, $n = 3$). The basic migratory activity of microglia, however, was only slightly influenced by DIDS treatment; moreover, DIDS treatment did not influence the viability of microglial cells determined by trypan blue staining (data not shown, $n = 3$).

The serine variant of CCL21 is the situ ligand in the brain

Two different CCL21 forms have been described recently (31). Both genes differ only in one amino acid at position 65 but are expressed in different tissues. The serine form is present in lymphoid organs in contrast to the leucine form of this chemokine (31). To investigate which form is induced in mouse brain upon ischemic brain damage, the full-length sequence of the CCL21 gene was determined by RT-PCR using the following primers: forward, #63, CAGCTCTGGTCTCATCCTCA; backward, #556, TGAACCACCCAGCTTGAAGT. Primers were designed according to the published sequence of CCL21 (AC: AF006637). PCR amplification using cDNA derived from either lymph nodes or ischemic brain gave rise to 493-bp product. Cloning and subsequent sequencing of the PCR product revealed no difference between the CCL21 expressed in lymph nodes and in ischemic mouse brain. In both cases, the serine form of CCL21 was found.

Discussion

The results presented here indicate that the chemokine CCL21 signals to microglial cells via the activation of CXCR3 and not CCR7, indicating that, in brain, CCL21 acts via a different receptor system than in lymphoid organs. Our observations support the activation of CXCR3 by CCL21 since 1) microglia from CXCR3 knockout animals were unresponsive to CCL21, 2) microglial cells obtained from CCR7 knockout animals respond to CCL21, 3) the CXCR3 ligand CXCL10 mimicked the response of CCL21, and 4) the CCR7 ligand CCL19 did not induce comparable effects as was found for CCL21. This is in line with the report that the classical receptor for this chemokine, CCR7, is not expressed by microglial cells (6). Thus, our results provide the first evidence that a CC chemokine activates a CXC chemokine receptor in a primary cell, supporting a previous finding in a recombinant system (32). It has been shown in transfected cells that human CCL21 did not activate human CXCR3 (33). Whether human microglia respond to stimulation with human CCL21 has not yet been addressed.

There are two different genes for CCL21 (31). Cloning studies indicated that the CCL21 gene inducibly expressed by neurons is the same as that in lymph nodes where it is constitutively expressed (31, 34). This indicates that the chemokine CCL21 which has been described this far as a constitutive chemokine in the periphery (35) is an inducible chemokine in the CNS. Taken together, these results indicate that the CNS is an organ where CCL21 chemokine signaling is different from that of the peripheral immune system.

The induction of inflammatory chemokines such as monocyte chemoattractant protein 1, macrophage-inflammatory proteins 1α and 1β, CXCL10, and monokine induced by IFN-γ in the CNS has been described in a variety of different brain diseases (36–41). Microglial cells respond to any type of CNS injury; therefore, chemokines are good candidates for signaling neural injury to microglia. Because it has been found that neurons express high levels of fractalkine and the corresponding receptor (CX3CR1) is expressed in microglia, a functional role of chemokines in the signaling from neurons to microglia has been suggested (42). Although microglial activation was unchanged in CX3CR1 knockout mice, other neuronal chemokines might contribute to the signaling (43). We have recently shown that damaged neurons in vivo and in vitro rapidly induce the expression of CCL21, and we therefore suggest that CCL21 signals neuronal damage to microglial cells (6). CCL21 has been demonstrated to induce Ca^{2+} signals and chemotaxis in cultured microglial cells (6). These cultured cells, however, are not a good model for resting, ramified microglial cells in brain tissue, which are the unactivated recipients receiving signals from injured neurons; the culture conditions transform microglial into activated (ameboid) phenotype. We provide first evidence that ramified microglial cells from acutely isolated brain tissue, which display a down-regulated immunophenotype, respond to CCL21 by the activation of a long lasting conductance increase. Thus, neuronal CCL21 via microglial CXCR3 may provide resting microglial cells with information on neuronal damage in their vicinity.

Because CCL21 triggered a conductance increase in both microglial cells in the slice and in culture, we used the cultured microglial cells as a model to study CCL21 signaling. Brief application (30 s) of CCL21 triggered a long lasting increase in a Cl^- conductance, which strongly changed the physiological properties of the cell for many minutes. We also provide first evidence that the activity of Cl^- channels and the chemotactic activity are linked; the Cl^- channel blocker DIDS impaired the CCL21-induced chemotaxis. Thus, the CCL21-induced Cl^- conductance could be an initial switch to alter microglial behavior after neuronal injury. There is more evidence that Cl^- channels influence cellular behavior, even in microglia. A long lasting Cl^--current can be evoked by membrane stretch and is thought to be required for the induction of ramification but not for maintaining the ramified shape of cultured microglial cells (44). Alternatively, Cl^- channels appear to be necessary for CSF-1-stimulated proliferation of rat microglia (45), given that Cl^- channels play a similar role in lymphocyte proliferation and control the activation of these cells (46). We could not yet find an effect of CCL21 on typical parameters of microglial activation, namely basal or LPS-induced synthesis of inflammatory cytokines (TNF-α, IL-6, IL-12), chemokines (macrophage-inflammatory protein 1α, monocyte chemoattractant protein 1) or nitric oxide (data not shown). Thus, the CCL21/CXCR3 signaling system is rather an indicator of neuronal damage to microglia than an induction system for microglial activation.

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